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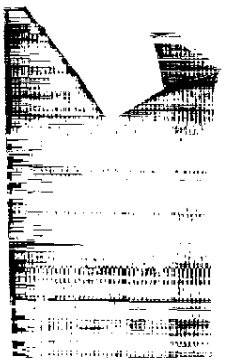

Haemoglobin adducts from aromatic amines and tobacco-specific nitrosamines in pregnant smoking and non-smoking women

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In non-smokers, haemoglobin adducts from 3- and 4-aminobiphenyl have been reported to arise mainly from exposure to environmental tobacco smoke (ETS). Therefore, the impact of self-reported smoking ($n = 27$) and exposure of non-smokers to ETS ($n = 78$) on haemoglobin adducts was studied in pregnant women from Homburg, Germany. In addition to 3- and 4-aminobiphenyl, adducts from seven monocyclic aromatic amines (aniline, *o*-, *m*-, and *p*-toluidine, 2,4-dimethylaniline, 2-ethylaniline and *o*-anisidine) and the adduct from tobacco-specific nitrosamines (4-hydroxy-1-(3-pyridyl)-1-butanone) were determined. Five of 78 self-reported non-smoking women had plasma cotinine levels and urinary cotinine/creatinine ratios indicative of active smoking. In the remaining 73 non-smokers cotinine/creatinine ratios correlated significantly with self-reported exposure to ETS. However, none of the haemoglobin adducts increased with increasing exposure to ETS or increasing cotinine/creatinine ratios. Although significantly elevated in smoking compared with non-smoking women, the mean haemoglobin adduct levels formed by tobacco-specific nitrosamines (54·7 ± 8·9 vs 26·7 ± 4·1 fmol g^{-1}, $p < 0{\cdot}001$), 3-aminobiphenyl (3·0 ± 0·5 vs 1·4 ± 0·1 pg g^{-1}, $p < 0{\cdot}001$), 4-aminobiphenyl (27·9 ± 3·4 vs 10·2 ± 0·7 pg g^{-1}, $p < 0{\cdot}001$), *o*-toluidine (289 ± 25 vs 237 ± 65 pg g^{-1}, $p < 0{\cdot}001$), *p*-toluidine (315 ± 32 vs 197 ± 13 pg g^{-1}; $p < 0{\cdot}001$), 2,4-dimethylaniline (25·5 ± 2·9 vs 18·6 ± 1·6 pg g^{-1}, $p < 0{\cdot}05$), had considerable overlappings ranges indicating lack of specificity as biomarkers to tobacco smoke exposure. Exposure to other as yet unknown environmental sources appears to be more significant than previously thought.

Keywords: haemoglobin adducts, aromatic amines, tobacco-specific nitrosamines, pregnant women, tobacco smoke, passive smoking.

Abbreviations: ABP, aminobiphenyl; HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone; ETS, environmental tobacco smoke; TSNA, tobacco-specific nitrosamines; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNN, N´-nitrosonornicotine; CCR, cotinine/creatinine ratio.

Introduction

Cigarette smoking is a risk factor for lung cancer and possibly other types of human cancer (Hoffmann and Hecht 1990). Intensive research on tobacco smoke carcinogenesis has suggested that polycyclic aromatic hydrocarbons, aromatic amines and tobacco-specific nitrosamines (TSNA) may be putative human carcinogens. These compounds are present in mainstream smoke and at equal or even higher amounts in sidestream smoke (Klus and Kuhn 1982, Grimmer *et al.*

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1987, Hoffmann *et al.* 1994, Grimmer and Schneider, 1995). Therefore, exposure to environmental tobacco smoke (ETS), i.e. 'passive' or involuntary smoking, may be a source of exposure to these compounds. Passive smoking has been suggested as a possible risk factor for lung cancer in several, but not all, epidemiological studies (US EPA 1992). In a meta-analysis of 30 studies the EPA estimated the relative risk for lung cancer in non-smoking women exposed to ETS to be 1·19 (90 % confidence interval 1·04–1·35). However, this risk estimate has been discussed controversially in the scientific community (Trédaniel *et al.* 1994, Gross 1995, Reynolds and Fontham 1995, Nilsson 1996, Armitage *et al.* 1997).

One major concern in epidemiological studies is the rate of misclassification of self-reported smoking status. The percent of female current smokers who deny current smoking varies from a low of < 2 % in the US and Germany (Fontham *et al.* 1994, Heller *et al.* 1998) to 24·3 % in Japan (Lee 1995). Classification according to self-reported ETS exposure may be even more erroneous (Tunstall-Pedoe *et al.* 1995, Barry 1997), indicating the need for biochemical validation of both self-reported smoking status and exposure to ETS. Cotinine is reported to be the most specific and most sensitive biomarker for both active smoking and exposure to ETS (Benowitz 1996). One major advantage of cotinine, the proximate metabolite of nicotine, is the nearly unique occurrence of nicotine in tobacco. However, cotinine is at best a valid biomarker for tobacco smoke exposure during the last 2–3 days and may not reflect accurately the exposure to other tobacco smoke components. The excretion of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and its *O*-glucuronide (NNAL-Glu), two metabolites of the TSNA 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are about two orders of magnitude lower in urine of non-smokers experimentally exposed to very high concentrations of cigarette smoke (Hecht *et al.* 1993) than reported in smokers (Meger *et al.* 1996). Therefore it is unlikely that significant amounts of NNAL and/or NNAL-Glu occur in urine of non-smokers exposed to ETS under real-life conditions (Tricker 1997).

The haemoglobin adducts of various compounds which are metabolized to electrophilic species have been proposed as suitable surrogate markers for exposure occurring over a period of several months (Skipper and Tannenbaum 1990). Adducts formed by polycyclic aromatic hydrocarbons, in particular benzo[*a*]pyrene, are not useful biomarkers of exposure to ETS because of high background exposure from the diet (Hattemer-Frey and Travis 1991) and traffic exhaust emissions (Pastorelli *et al.* 1996). Adducts formed by the aromatic amines 3- and 4-aminobiphenyl (ABP) have been proposed as rather specific biomarkers for tobacco smoke exposure (Bryant *et al.* 1987, Maclure *et al.* 1989, Bartsch *et al.* 1990, Skipper and Tannenbaum 1990). Although a significant correlation between 4-ABP haemoglobin adduct levels and exposure to ETS has previously been reported in non-smoking pregnant women (Hammond *et al.* 1993), most studies report only weak correlations suggesting that additional unidentified environmental sources of ABP contribute to haemoglobin adduct levels (Maclure *et al.* 1989, Falter *et al.* 1994). Elevated levels of haemoglobin adducts from monocyclic aromatic amines have been reported in smokers (Bryant *et al.* 1988), but no information is available on the contribution of exposure to ETS to these adducts in non-smokers.

A single haemoglobin adduct formed by the two tobacco-specific nitrosamines, N′-nitrosonornicotine (NNN) and NNK could, in theory, be an ideal biomarker of tobacco smoke exposure (Hecht 1994, Richter 1996). Metabolism of both nitrosamines give rise to a haemoglobin adduct releasing 4-hydroxy-1-(3-pyridyl)-

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1-butanone (HPB) upon mild alkaline hydrolysis. According to the NNK and NNN levels in mainstream smoke and ETS (Hoffmann *et al.* 1994) as well as the urinary NNAL excretion rates discussed above, smokers should have HPB adduct levels at least two orders of magnitude higher than ETS-exposed non-smokers. However, current studies report significant overlapping ranges of HPB adduct levels in both smokers and non-smokers because only about 20 % of active smokers appear to have elevated adduct levels (Carmella *et al.* 1990, Falter *et al.* 1994).

In this study, cotinine, a short term biomarker of tobacco smoke exposure, and haemoglobin adducts from aromatic amines and tobacco-specific nitrosamines were determined to investigate (1) the specificity of haemoglobin adducts as biomarkers of smoking status and ETS exposure, and (2) whether exposure to ETS contributes to background haemoglobin adduct levels in non-smoking pregnant women.

Materials and methods

Study subjects

Caucasian women admitted to the Gynecological Clinic of the Homburg/Saar University during autumn 1992 to spring 1994 for labour and delivery were recruited. On admission to the clinic the study was explained and those willing to participate signed a written consent form approved by the Ethics Committee of the University of Homburg. Altogether, 105 women were enrolled, 78 declared themselves to be non-smokers and 27 claimed to be active smokers. All subjects completed a validated investigator-administered questionnaire to assess general health, life style, occupation, smoking habits and exposure to ETS. The sociodemographic data are summarized in table 1. Based on the subject's living conditions, exposure to ETS was stratified into four categories, namely no ETS exposure (score 0) or low (score 1), moderate (score 2) or heavy (score 3) exposure to ETS (figure 1). The highest ETS exposure category was given to subjects who had a smoking partner who continued to smoke during the pregnancy, as well as exposure to ETS from other household members and/or people at the work place.

Collection of samples

A blood sample (20 ml) was collected into EDTA-treated vacutainers and a spot urine sample (3 ml) was obtained during admission for labour. After centrifugation, plasma was separated from blood cells which were divided into two equal parts and washed twice with 8 ml of saline. All samples were coded and stored at –20 to –80 °C.

Determination of haemoglobin adducts

Haemoglobin adducts from aromatic amines and tobacco-specific nitrosamines were determined as previously described (Kutzer *et al.* 1997). Briefly, haemoglobin solutions obtained after centrifugation of lysed red blood cells were dialysed against deionized water. Haemoglobin content was determined by Drabkin's assay (Sigma, Deisenhofen, Germany). After addition of internal standards, 40 pg D_5-HPB, 40 pg D_9-4-ABP and 80 pg D_5-aniline, adducts were released by mild base-catalysed hydrolysis. Extraction, clean-up and concentration were performed by a one-step procedure using C_{18} cartridges. Two equal parts of the extract were derivatized separately with pentafluoropropionic anhydride and pentafluorobenzoylchloride for aromatic amines and TSNA-derived HPB, respectively. Analysis was performed by capillary gas chromatography–mass spectrometry with negative chemical ionization and selected ion monitoring. The analytical limit of detection was 0·5–2 pg adduct per g haemoglobin using a 10 ml aliquot of blood. All samples were analysed blind in duplicate. Two blank water samples without addition of internal standards were analysed each day to control for background contamination.

Analysis of cotinine and creatinine

Cotinine in plasma and urine was determined by a radioimmunoassay according to the method of Langone *et al.* (1973) with modifications by Haley *et al.* (1983). Creatinine in urine was determined by the Jaffé method using a commercial test kit (Merck, Darmstadt, Germany).

Statistical analysis

Comparison of group means was performed by a *U*-test according to Mann–Whitney using WinSTAT (G. Greulich Software, Staufen, Germany). All other analyses were conducted by use of the SAS 6·07 Statistics Software Package (SAS Institute Inc., Cary, NC).

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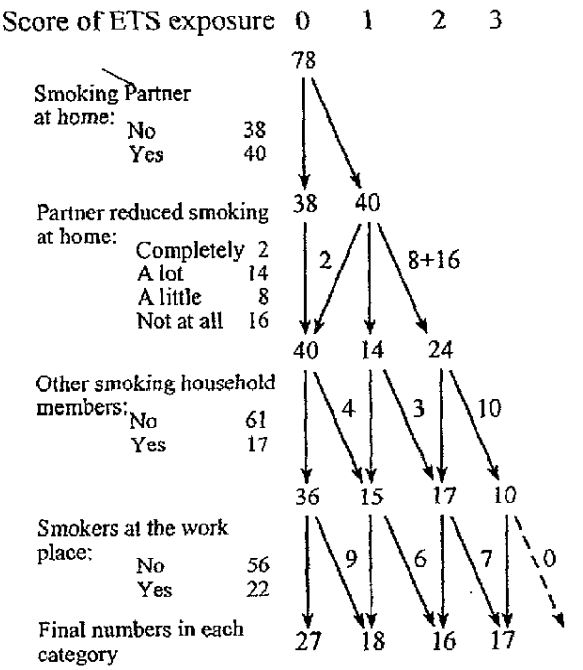

Figure 1. Score of ETS exposure in non-smoking pregnant women: 0 = no; 1 = little; 2 = moderate; 3 = heavy exposure. Numbers adjacent to the oblique arrows indicate the number of women moved to a higher or lower score category according to their self-reported exposure to ETS.

Results

Cotinine in plasma and urine

Five of 78 self-reported non-smoking women (6·4 %) had plasma concentrations (36, 38, 90, 93 and 150 ng ml^{-1}) and urinary cotinine/creatinine ratios (CCR; 643, 1432, 2235, 1077 and 4753 ng mg^{-1}) indicative of current smoking. They were omitted from statistical evaluation (table 2; figure 2). Interestingly, four of these five women declared themselves as heavily and one woman as moderately ETS exposed (scores 2 and 3). The remaining 73 non-smokers had plasma cotinine levels which were either between 0·5 and 4 ng ml^{-1} ($n = 15$) or not detectable ($< 0·5$, $n = 58$). Six women had a CCR of > 50 ng mg^{-1}, two claimed to be moderately and four heavily exposed to ETS. Evaluation of CCR by non-parametric Kruskal–Wallis test ($p < 0·005$) indicated a highly significant difference between the four different ETS exposure categories confirming in this situation the validity of self-reported ETS exposure (table 2; figure 2).

Four of 27 self-reported smokers had plasma cotinine levels ($< 0·5$, $< 0·5$, 1·0 and 2·0 ng ml^{-1}) and CCR (5·5, 5·1, 54 and 56 ng mg^{-1}) indicative of no smoking during the 2–3 days prior to sampling. Because of the long half-life of haemoglobin adducts, these subjects were not omitted from the statistical evaluation (table 2). All other smokers had plasma cotinine levels > 10 ng ml^{-1} and CCR > 250 ng mg^{-1}. CCR was significantly higher in smokers as compared with non-smokers, 2525 ± 389 vs $18·9 \pm 3·6$ ng mg^{-1} ($p < 0·0001$). Plasma cotinine levels in smoking pregnant women averaged 103 ± 16 ng ml^{-1}. CCR and plasma cotinine were strongly correlated according to Pearson analysis ($p < 0·0001$; table 3).

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Table 1. Selected sociodemographic data of the study population. Five of 78 self-reported non-smoking women had CCR values > 250 ng mg^{-1} indicating active smoking and were removed from the analysis to leave 73 confirmed non-smokers.

Variable	Self-reported non-smokers ($n = 78$)	Confirmed non-smokers ($n = 73$)	Smokers ($n = 27$)
Age (years)	29·6 ± 5·2	29·5 ± 5·4	28·7 ± 6·2
(min./max.)	(18·6/43·1)	(18·6/43·1)	(20·5/39·1)
Marital status; number (%)			
Married	67 (85·9)	64 (87·7)	18 (66·7)
Living with a partner	6 (7·7)	5 (6·9)	7 (25·9)
Singles	5 (6·4)	4 (5·3)	2 (7·4)
Education; number (%)			
High school or above	41 (52·6)	39 (53·5)	8 (32·0)*
< High school	37 (47·4)	34 (46·6)	17 (68·0)

[a] Two missing values ($n = 25$).

Haemoglobin adducts in smoking and non-smoking pregnant women

The mean levels of haemoglobin adducts are summarized in table 2. For better comparison with published data results from aromatic amines are given in pg g^{-1} haemoglobin whereas the concentration of the TSNA adduct HPB is expressed as fmol g^{-1} haemoglobin (1 fmol corresponds to 0·165 pg). Generally, adduct levels from toluidines and aniline were one and two orders of magnitude higher than those found for 4-ABP, 2-ethylaniline, 2,4-dimethylaniline and *o*-anisidine. The 3-ABP adduct had by far the lowest concentrations and was below the limit of detection (0·5 pg g^{-1}) in 11 of 73 non-smokers and in two of 27 smokers. The HPB adduct was also below the limit of detection (6 fmol g^{-1}) in 11 of 73 non-smokers and in one smoker.

Five of nine aromatic amines, 3-ABP (2·2-fold), 4-ABP (2·7-fold) *o*-toluidine (1·2-fold), *p*-toluidine (1·6-fold) and 2,4-dimethylaniline (1·4-fold) as well as HPB (2·0-fold) were significantly elevated in smokers as compared with non-smokers. None of these haemoglobin adducts appeared to be a specific biomarker for active smoking because of the considerable overlapping ranges. The 1·3-fold difference found for 2-ethylaniline did not reach statistical significance. Aniline, *m*-toluidine and *o*-anisidine were detected in all blood samples at similar levels in both smoking and non-smoking women.

No significant positive correlations were found between the CCR and any haemoglobin adduct in non-smokers (table 3). Adducts from monocyclic aromatic amines tended to decrease with increasing CCR and this trend was significant for *m*-toluidine ($p < 0·05$). In contrast, weak positive correlations were obtained for smoking pregnant women, approaching significance for aniline ($p = 0·0565$), 3-ABP ($p = 0·0572$), 4-ABP ($p = 0·0691$) and HPB ($p = 0·0574$). Similar positive correlations in Pearson analysis were seen for plasma cotinine in smokers which were significant for aniline, *o*-toluidine, 3- and 4-ABP ($p < 0·05$) but not for HPB ($p = 0·0547$) and *p*-toluidine ($p = 0·0556$). With the exception of *o*-toluidine, adducts from monocyclic aromatic amines were highly correlated with each other in non-smoking but less so in smoking pregnant women. Linear regression showed a very high correlation between adducts from 2-ethyl- and 2,4-dimethylaniline in both smokers and non-smokers (figure 3). In contrast, the correlation between 3-

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Table 2. *Effect of smoking status on haemoglobin adducts (EA = ethylaniline, DMA = dimethylaniline, ABP = aminobiphenyl, HPB = 4-hydroxy-1-(3-pyridyl)-1-butanone) and urinary cotinine/creatinine ratio (CCR) in pregnant women; score 0, 1, 2 and 3 represents no, low, moderate and heavy ETS exposure; mean ± SE (min./max.).*

Smoking status	Aniline (pg g^{-1})	*o*-Toluidine (pg g^{-1})	*m*-Toluidine (pg g^{-1})	*p*-Toluidine (pg g^{-1})	2-EA (pg g^{-1})	2,4-DMA (pg g^{-1})	*o*-Anisidine (pg g^{-1})	3-ABP (pg g^{-1})	4-ABP (pg g^{-1})	HPB (fmol g^{-1})	CCR (ng mg^{-1})
Active smoking	1190 ± 122	289 ± 25[b]	672 ± 666	315 ± 32[b]	24·9 ± 3·3	25·5 ± 2·9[a]	80·2 ± 11·9	3·0 ± 0·5[b]	27·9 ± 3·4[c]	54·7 ± 8·9[b]	2525 ± 389[b]
(n = 27)	(305/2900)	60/550)	(178/1390)	(101/905)	(4·5/80·0)	(5·0-/61·0)	(12·0/256)	(0·0/14·0)	(6·0/65·0)	(0/225)	(5/5284)
Non-smoking	1240 ± 97	237 ± 65	706 ± 55	197 ± 13	19·1 ± 1·6	18·6 ± 1·6	97·2 ± 8·7	1·4 ± 0·1	10·2 ± 0·7	26·7 ± 4·1	18·9 ± 3·6
(n = 73)	(194/5265)	(24/4630)	(165/2551)	(38/575)	(4·0/63·0)	(3·0/58·0)	(10·0/304)	(0·0/6·0)	(4·0/36·0)	(0/229)	(1·7/180)
ETS exposure											
Score 0	1297 ± 160	356 ± 172	808 ± 90	222 ± 26	24·6 ± 3·4	21·6 ± 3·0	117·7 ± 16·8	1·2 ± 0·2	10·6 ± 1·5	35·9 ± 9·8	6·5 ± 1·3
(n = 27)	(412/4410)	(28/4630)	(165/2000)	(65/575)	(4/0/63/0)	(3·0/57·0)	(26·0/304)	(0·0/3·0)	(4·0/36·0)	(0/229)	(1·7/33·2)
Score 1	1541 ± 273	179 ± 22	813 ± 126	204 ± 24	17·2 ± 2·9	18·2 ± 3·2	106·3 ± 18·0	1·8 ± 0·3	10·6 ± 1·2	20·2 ± 4·4	15·8 ± 2·6
(n = 18)	(194/5265)	(85/381)	(234/2551)	(104/424)	(4·5/51·0)	(5·5/58·0)	(26·0/244)	(0·0/6·0)	(6·0/27·0)	(0/75·0)	(2·4/39·6)
Score 2	955 ± 113	167 ± 23	620 ± 128	174 ± 20	15·9 ± 2·5	17·8 ± 3·3	75·5 ± 14·5	1·3 ± 0·2	9·3 ± 0·8	25·4 ± 6·4	17·8 ± 5·3
(n = 14)	(528/1714)	(94/360)	(184/2110)	(91/375)	(6·0/35·0)	(6·0/53·0)	(21·0/217)	(0·0/3·5)	(6·0/16·5)	(0/84·0)	(2·8/63·3)
Score 3	1026 ± 125	148 ± 18	458 ± 70	164 ± 17	14·2 ± 2·5	14·2 ± 2·5	67·6 ± 11·9	1·2 ± 0·2	9·6 ± 0·8	18·8 ± 3·7)	46·8 ± 14·9
(n = 14)	(239/2099)	(24/264)	(170/1045)	(38/260)	(6·0/38·0)	(6·0/35·0)	(10·0/157)	(0·0/3·0)	(6·0/16·0)	(0/45·0)	(2·1/180)
Kruskal–Wallis test	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	$p < 0·005$

[a,b] Significantly different from non-smokers (Mann–Whitney U-test); $p < 0·05$ ([a]), $p < 0·001$ ([b]).

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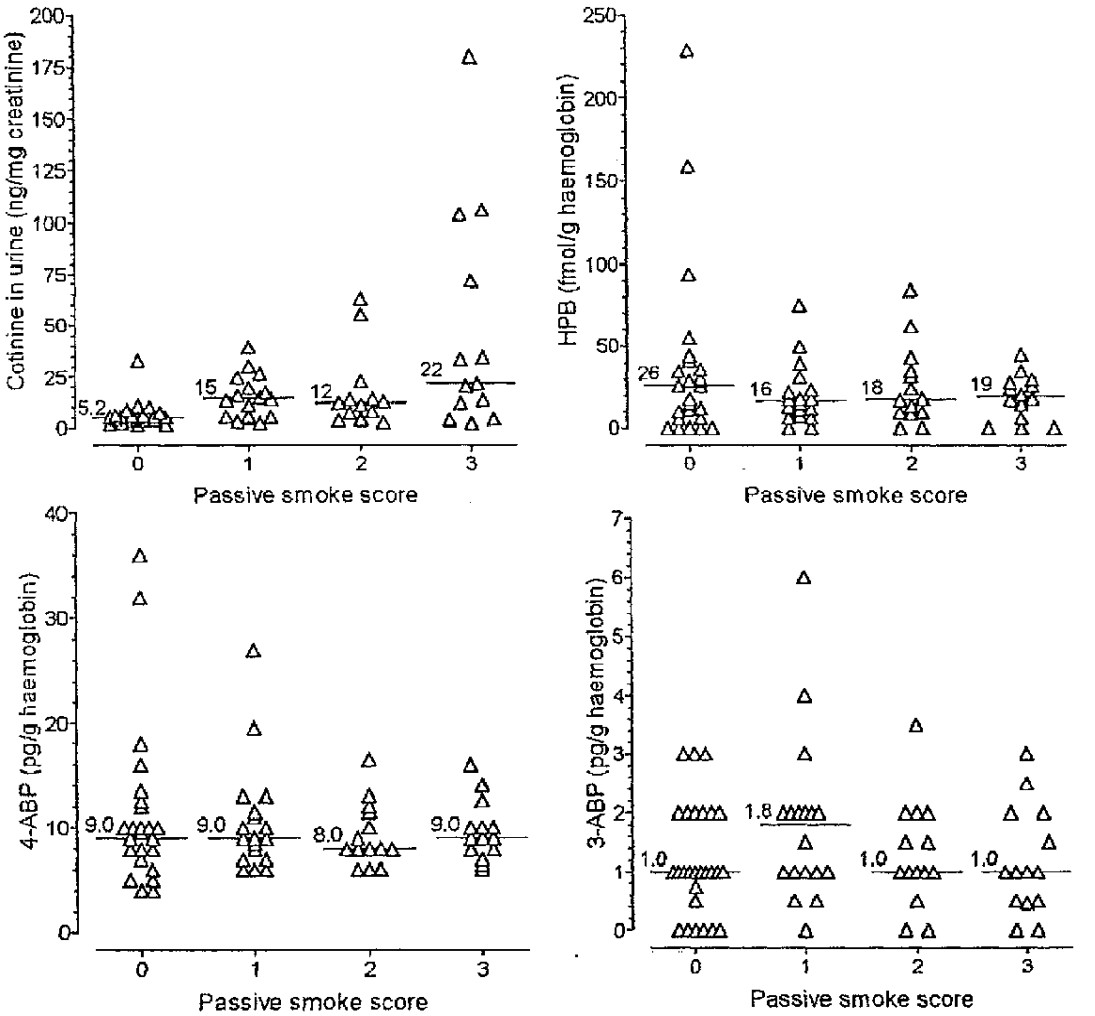

Figure 2. Correlation between self-reported exposure to ETS and urinary cotinine as well as haemoglobin adducts from TSNA, 4-ABP and 3-ABP in non-smoking pregnant women. Bars represent median values.

and 4-ABP was stronger in smokers ($p = 0{\cdot}0032$) than in non-smokers ($p = 0{\cdot}0431$). Correlations between adducts of monocyclic and bicyclic amines were rather weak and usually not significant. The TSNA adduct HPB correlated only weakly with 4-ABP in smoking ($p = 0{\cdot}0501$) but strongly in non-smoking women ($p < 0{\cdot}0001$).

Self-reported exposure to ETS did not correlate with any of the haemoglobin adducts (figure 2; table 2). No significant differences (non-parametric ANOVA analyses) were found between the adduct levels when classified according to the four different ETS exposure categories.

Discussion

Cotinine is usually a reliable marker for current active smoking (Lee 1987, US EPA 1992, Kemmeren *et al.* 1994). However, in the present investigation 4 of 27 women (14·8 %) who declared themselves as active smokers would have been misclassified as non-smokers. On the other hand, 5 of 78 self-reported non-

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Table 3. Pearson correlation coefficients between haemoglobin adducts (EA = ethylaniline, DMA = dimethylaniline, ABP = aminobiphenyl, HPB = 4-hydroxy-1-(3-pyridyl)-1-butanone), urinary cotinine/creatinine ratio (CCR) and cotinine in plasma (P-Cot) from pregnant women. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

	Aniline	*o*-Toluidine	*m*-Toluidine	*p*-Toluidine	2-EA	2,4-DMA	*o*-Anisidine	3-ABP	4-ABP	HPB	CCR
Smoking pregnant women ($n = 27$; lower left triangle)					Non-smoking pregnant women ($n = 73$; upper right triangle)						
Aniline		0·006	0·512****	0·438****	0·489****	0·620****	0·576****	0·287**	0·012	0·043	−0·093
o-Toluidine	0·612***		0·018	−0·057	−0·005	0·002	0·003	−0·034	0·005	0·058	−0·079
m-Toluidine	0·265	0·358*		0·536****	0·633****	0·735****	0·593****	0·207*	0·229*	0·304**	−0·239*
p-Toluidine	0·706****	0·709****	0·230		0·585****	0·594****	0·579****	0·276**	0·296**	0·305**	−0·068
2-EA	0·143	0·252	0·553**	0·236		0·909****	0·664****	0·246*	0·137	0·417***	−0·132
2,4-DMA	0·177	0·249	0·600***	0·259	0·915****		0·673****	0·199*	0·025	0·253*	−0·193
o-Anisidine	0·193	0·065	0·434*	0·264	0·375*	0·333*		0·228*	0·024	0·079	−0·145
3-ABP	0·052	0·010	−0·074	0·026	0·048	0·065	−0·243		0·202*	0·156	0·063
4-ABP	−0·047	−0·005	−0·078	−0·036	0·243	0·140	−0·201	0·512**		0·548****	0·033
HPB	−0·002	0·063	−0·237	−0·085	−0·102	−0·235	−0·207	0·010	0·323		−0·067
CCR	0·318	0·255	0·082	0·249	0·139	0·183	−0·122	0·317	0·299	0·317	
P-Cot	0·408*	0·405*	−0·016	0·320	0·026	0·081	−0·225	0·377*	0·390*	0·321	0·934****

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smoking women (6·4 %) had cotinine levels indicating current smoking. This percentage of misclassification would have been higher if a lower cut-off value for CCR had been applied (Fontham *et al.* 1994, de Waard *et al.* 1995, Riboli *et al.* 1995). Three additional non-smoking women reporting high exposure to ETS (score 3) had a CCR of >100 ng mg^{-1} while two reporting moderate exposure to ETS (score 2) had a CCR of >50 ng mg^{-1}. Applying these cut-off points, the misclassification rate would have increased to 10·3 % and 12·8 %, respectively. These misclassification rates are higher than those usually reported in European (Heller *et al.* 1998) and US populations (Fontham *et al.* 1994). One possible explanation is that pregnant women are not as willing to admit to active smoking as the general population. A similar observation has been reported by Apseloff *et al.* (1994) who found 16 % of smokers among self-reported non-smokers enrolling in clinical trials designed for non-smokers.

Cotinine in body fluids has been reported to be the best available biomarker of ETS exposure at present (Benowitz 1996). In the present study cotinine in plasma was detectable in 15 of 73 non-smokers and all samples of spot urine. A significant correlation was found between self-reported exposure to ETS and CCR. On average, pregnant women reporting no exposure to ETS (score 0; CCR: $6·5 \pm 1·3$ ng mg^{-1}, $n = 27$) had three to four times lower CCR than women reporting exposure to ETS (scores 1, 2 and 3; CCR: $26·0 \pm 5·3$ ng mg^{-1}, $n = 46$). Much lower differences were obtained within the arbitrary scores of low, moderate or high ETS exposure (table 2). Omission of three non-smoking women with CCR >100 would have lowered the mean CCR in score 3 to 21·8 ng mg^{-1}. Because of significant overlapping ranges no reliable cut-off point could be defined, which would distinguish non-smokers with or without self-reported exposure to ETS (figure 2).

The most striking result of the present investigation is the complete absence of correlations between aromatic amine and TSNA haemoglobin adducts and self-reported exposure to ETS. There was no correlation between ABP adducts with either self-reported ETS exposure or cotinine levels. Contrary to this, previous studies have reported small ETS exposure-related increases of borderline statistical significance for haemoglobin adducts from 3- and 4-ABP (Maclure *et al.* 1989, Bartsch *et al.* 1990, Hammond *et al.* 1993). Hammond *et al.* (1993) in a similar, but smaller, study found statistically higher levels of 4-ABP haemoglobin adducts in pregnant women at term with either high or low ETS exposure ($27·8 \pm 1·4$ vs $17·6 \pm 2·4$ pg g^{-1}, $p = 0·027$). The women were categorized according to levels of airborne nicotine collected with personal samplers over 1 week during the third trimester. Although a strong correlation ($r = 0·87$) between self-reported ETS exposure and the amount of nicotine on the sampler was reported, misclassification of active smoking women as seen in our study cannot be excluded either in the study of Hammond *et al.* (1993). Being aware of the control of their smoking habit, active smoking women declaring themselves as non-smokers may have either resisted smoking during the period of air nicotine sampling or had not used the air sampling device correctly. The baseline 4-ABP adduct levels in pregnant non-smoking women from Homburg ($10·2 \pm 0·7$ pg g^{-1} haemoglobin) were about half of the levels found in pregnant women from Worcester, MA ($22 \pm 1·3$ pg g^{-1}), Hammond *et al.* 1993) or Louisville, KY ($18·3 \pm 1·5$ pg g^{-1}, Myers *et al.* 1996). Although this may be due to variations in the analytical methods, our own investigations indicate regional differences occur in haemoglobin adduct levels. 4-ABP adduct levels in non-smokers from rural areas of Bavaria (15 ± 3 pg g^{-1}) are

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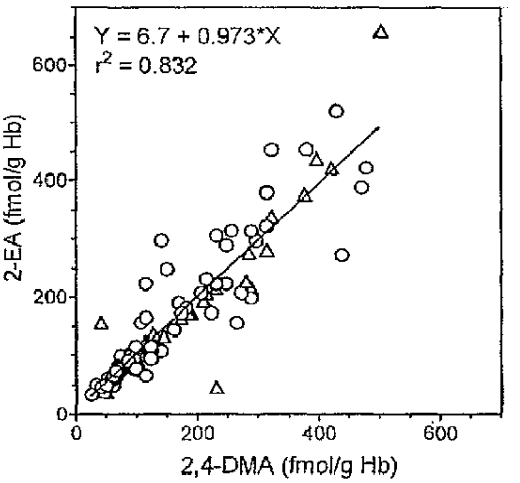

Figure 3. Linear regression analysis between haemoglobin adducts from 2, 4-dimethylaniline (2, 4-DMA) and 2-ethylaniline (2-EA) in smoking (△; $n = 27$) and non-smoking (●; $n = 73$) pregnant women.

significantly lower ($p < 0{\cdot}01$) than those found in non-smokers (29 ± 4 pg g^{-1}) who lived in Munich (Falter *et al.* 1994). The Gynecological Clinic of the Homburg/Saar University is situated in a small town of southern Germany with a large percentage of patients from rural areas. Whether the differences in baseline 4-ABP haemoglobin adduct levels are due to other unidentified sources of 4-ABP exposure (Hammond *et al.* 1993, Falter *et al.* 1994, Myers *et al.* 1996), differences in polymorphic expression of enzymes involved in 4-ABP metabolism and binding to haemoglobin (Bartsch *et al.* 1990, Vineis *et al.* 1994, Yu *et al.* 1994, 1995, Landi *et al.* 1995) or simply differences in analytical methodology, remains uncertain.

The concentrations of monocyclic aromatic amine haemoglobin adducts were in the same range as reported for smokers and non-smokers (Bryant *et al.* 1988, Falter *et al.* 1994). The small but significant contribution of smoking to *o*-toluidine, *p*-toluidine and 2,4-dimethylaniline adducts and the high correlation between different monocyclic amine adducts in smokers are in agreement with another study (Ronco *et al.* 1990). Our study is the first to report the relationship between monocyclic amine adducts and exposure to ETS in non-smokers. No indication of an ETS-related increase in adduct levels in non-smokers was found; although not significant, a tendency for decreasing adduct concentrations with increasing self-reported exposure to ETS was noted for all adducts except for aniline, 3-ABP and 4-ABP (table 2). Although the occurrence of monocyclic aromatic amines in tobacco smoke is well documented (Patrianakos and Hoffmann, 1979, Luceri *et al.* 1993, Grimmer and Schneider 1995), little is known about other environmental sources. The high correlations between adduct levels of several monocyclic aromatic amines (table 3), in particular 2,4-dimethylaniline and 2-ethylaniline with a slope of ~1 in both, smokers (slope = 1·054; $r^2 = 0{\cdot}838$) and non-smokers (slope = 0·946; $r^2 = 0{\cdot}826$; figure 3), are indicative of a common environmental source. The lack of correlation between adducts from *o*-toluidine and other monocyclic aromatic amines in non-smokers suggests an alternative environmental source for this compound. The unexpected observed negative correlation between adducts from monocyclic aromatic amines and exposure to ETS in non-smokers cannot be explained by current knowledge of their

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metabolism in experimental animals (DeBord *et al.* 1992) and man (Lang and Kadlubar 1991).

The results for the HPB-releasing haemoglobin adduct from TSNA confirm its weak specificity for exposure to tobacco smoke (Carmella *et al.* 1990, Falter *et al.* 1994). Contrary to our expectation, the concentrations of the TSNA-derived adduct decreased rather than increased with increasing self-reported ETS exposure. As discussed in detail elsewhere (Richter 1996), no explanation can be offered at present for these findings which are in sharp contrast to results obtained with urinary NNAL as biomonitor of NNK uptake (Hecht *et al.* 1993, Meger *et al.* 1996). Besides induction of TSNA detoxification and/or inhibition of TSNA activation in smokers (Carmella *et al.* 1990, Richter and Tricker, 1994, Lee *et al.* 1996) the existence of additional precursors to the HPB adduct from sources other than tobacco cannot be neglected. Therefore the validity of the HPB adduct as a biomarker of tobacco smoke is doubtful.

In summary, the results highlight the importance of selecting a valid biomarker in studies designed to assess exposure to ETS. Our study confirms the short term validity of cotinine (Benowitz 1996) and clearly shows that haemoglobin adducts from aromatic amines and TSNA are too non-specific for the quantitation of ETS exposure in non-smokers. Whereas urinary cotinine increased significantly with increasing self-reported exposure to ETS, no increase could be determined for the measured haemoglobin adducts.

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